

Abrogation of Adriamycin-Induced Cardiotoxicity by Selenium in Rabbits

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Adriamycin-induced cardiomyopathy in rabbits was produced by intravenous injections of the drug with a short therapeutic schedule (3 mg/kg body wt administered as four intermittent doses). Animals receiving selenium supplementation of Adriamycin showed preservation of the normal pattern of the heart histologic picture. The protective effect of selenium was accompanied by increased selenium levels in the plasma and the heart muscle. An eventual interaction between the antitumor effect of Adriamycin and the protective ef-

fect of selenium was ruled out by *in vitro* experiments using the L₁₂₁₀ cell line. Selenium did not abrogate the antiproliferative effect of Adriamycin when the cells were treated simultaneously with both agents. The results from this study indicate that Adriamycin-induced cardiotoxicity could be prevented by selenium if the animals were pretreated with selenium, rather than simultaneous administration of both agents. The mechanism of this effect is not entirely understood. (Am J Pathol 1987, 126:376-383)

ADRIAMYCIN (Adria Laboratories, Inc., Columbus, Ohio) is an antitumor antibiotic of the anthracycline group with a broad spectrum of therapeutic activity.¹ It has been shown that this drug produces a dose-related cardiomyopathy that can compromise its clinical use.²

Chronic administration of Adriamycin in rabbits produces characteristic cardiomyopathy manifested by myofiber degeneration with vacuolization followed by necrosis and interstitial fibrosis.^{3,4} These findings are identical to those seen in humans.^{5,6} In humans, these changes are observed more frequently when the dose of Adriamycin exceeds 500 mg/sqm.^{2,6} The rabbit heart appears to be more sensitive to Adriamycin than those of other species.⁷ Early histologic changes in humans have been reported at cumulative doses in excess of 120 mg/sqm.⁸ For better evaluation of the changes observed in the heart endomyocardial biopsy, a graded histologic system has been proposed.⁵

In order to prevent the occurrence of cardiomyopathy during therapy with Adriamycin, several methods using antioxidants have been investigated.^{6,9,10} The rationale behind the proposed modalities is acceptance of the theory that the histologic changes ob-

served in the heart are a result of free radicals released after administration of Adriamycin.¹¹ Accordingly, free radical scavengers have been used in animals and humans to protect the heart from this serious damage.^{9,10,12}

In this communication we report the results from experiments in rabbits using sodium selenite to prevent Adriamycin-induced cardiomyopathy.

Materials and Methods

Animals and Diet Supplementation

Sixteen female New Zealand white rabbits weighing 2.5 kg each were divided into four experimental groups. All groups were given commercial rabbit diet *ad libitum* containing 0.193 µg selenium/g body wt. The first group (Group I) received water *ad libitum*. The second group (Group II) was given selenium-supplemented water *ad libitum* with 4.34 µg sodium

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selenite (Sigma Chemical Co., St. Louis, Mo) per liter (2 ppm). The third group (Group III) was given water and was designated to receive Adriamycin. The fourth group (Group IV) received selenium-supplemented water and Adriamycin.

Experimental Groups and Treatment

Group I served as nontreated controls. Group II received the selenium-supplemented water from Day 1 until sacrifice on Day 19. Group III received normal water plus Adriamycin, 3.0 mg/kg body wt intravenously, on Days 8, 10, 14, and 18. Group IV received selenium supplementation plus intravenous Adriamycin, 3.0 mg/kg body wt, on Days 8, 10, 14, and 18. One day after the last dose of Adriamycin (Day 19) all animals were sacrificed, and plasma and heart samples were collected.

Transmission Electron Microscopy Preparation

After the animals were sacrificed, the heart was divided into atrial and ventricular portions, which were processed separately.

Sections of myocardial tissue were taken from the free wall of the left ventricle of each heart. A total of 13 hearts were acceptable for electron microscopy preparations. Samples were placed in phosphate buffer, pH 7.4, and diced into 1-cu mm blocks, which were fixed in 2.5% glutaraldehyde in phosphate buffer, pH 7.4. Following postfixation in 1% osmium tetroxide in phosphate buffer, pH 7.4, the material was dehydrated in the conventional manner, and 10 blocks from each heart were embedded in Epon-Araldite. Sections (2 μ m) were stained with toluidine blue, and a quantitative analysis was performed with modified Billingham's grades⁵: 0, normal myocardial ultrastructure; 0.5, isolated myocytes affected; 1, occasional myocytes affected by distended sarcotubular system and/or early myofibrillar loss and damage to 5% of all cells in 10 plastic blocks; 1.5, changes similar to those in Grade 1 but with damage 6–15% of all cells in 10 plastic blocks; 2.5, many myocytes, 26–35% of all cells in 10 plastic blocks, affected by vacuolization and/or myofibrillar loss; 3.0, severe and diffuse myocyte damage (35% of all cells in 10 plastic blocks) affected by vacuolization and/or myofibrillar loss.

Ten 600 Å sections were cut from each block and stained with uranyl acetate and lead citrate. Transmission electron microscopy was performed with a Philips 201 and a Philips 301 at 60 kv. All morphologic examinations were performed as a blind study by two independent observers.

Selenium Determination

Unfixed heart muscle was homogenized in Krebs–Ringer phosphate buffer, and the cytosol fraction was obtained with the use of centrifugation at 105,000g at 4 C. This fraction and plasma from heparinized whole blood were analyzed for selenium by the method of Whetter and Ullrey.¹³ The method of Lowry et al was used for protein determination.¹⁴

In Vitro Studies

The L₁₂₁₀ leukemia cell line was obtained from Dr. M. Chirigos, NCI. The culture was maintained in RPMI 1640 medium supplemented with 50 μ g/ml gentamycin, 3 μ g/ml Fungizone, and 15% heat-inactivated fetal bovine serum in 5% CO₂ at 37 C. Cultures were passaged twice a week with 1×10^6 viable cells/ml as the inoculum. Viable cell counts were determined by the Trypan blue exclusion technique. The antiproliferative effect of Adriamycin in the presence of selenium was determined. The selected cytotoxic dose of Adriamycin was 100 ng/ml, and the selenium dose was 0.5 μ g/ml. The fetal bovine serum which was from one stored batch contained 0.08 μ g selenium/ml. Only 15% of this amount was used in the culture medium. L₁₂₁₀ cells in exponential growth were diluted to $0.5\text{--}0.75 \times 10^6$ viable cells/ml in tissue culture medium. Twenty-four hours after seeding the tissue culture flasks, the selected doses of agents were added and incubated until the end of the experiment. Daily viable cell counts were performed for 7 days. For determination of intracellular selenium, the incubation mixture was centrifuged 1000 rpm for 10 minutes at 4 C. This procedure took place 24 hours after the addition of 0.5 μ g selenium/ml. In a separate experiment, cells were washed with PBS.

Statistical Analysis

One-way analysis of variance and the Tukey test were used for assessment of the data.¹⁵

Results

The results from the ultrastructural studies using transmission electron microscopy are presented in Figures 1–4. The control group fed the regular diet showed preserved subcellular structures (Figure 1). The appearance of myocardium of rabbits on selenium alone (Figure 2) is similar to that of the control nontreated group. The electron micrograph from rabbit heart injected with four doses of Adriamycin (Group III) revealed changes in the myofibrils, mito-

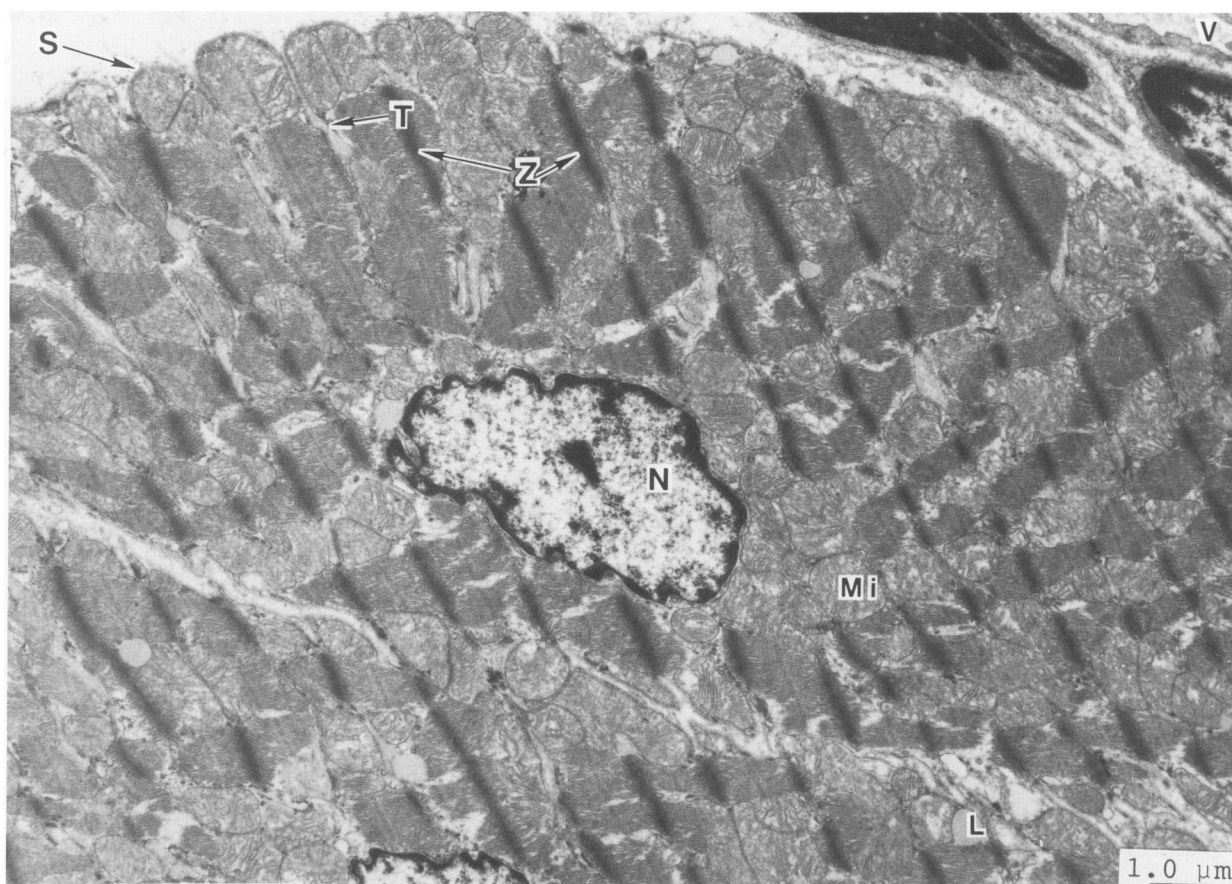


Figure 1 — Transmission electron microscopy of myocardium of control rabbit (normal). ($\times 4000$) Myofibers contain straplike myofibrils with prominent Z bands at regular intervals. Invagination of the sarcolemma (S) into the myofiber forms the T (T) tubules. Mitochondria (M) interspersed in regular rows between the myofibrils (interfibrillar) and around the nucleus (N) (perinuclear). Occasional lipid droplets (L) present in relation to the mitochondria.

chondria, and sarcoplasmic reticulum (Figure 3). An electron micrograph from the heart of a rabbit supplemented with selenium before administration of Adriamycin showed preservation of the myofibrils (Figure 4). The picture was similar to the findings seen in the heart of the control animals (Figure 1).

The results of the quantitation of the lesions are presented in Table 1. Statistical evaluation using one-way analysis of variance showed that the Adriamycin group (Group III) was significantly different from the other three groups. No significant difference was found among Groups I, II, and IV.

In order to confirm absorption and utilization of selenium by supplemented animals, we measured the selenium levels in the plasma and hearts of all experimental groups. The results of these studies are presented in Figures 5 and 6. There was no statistical difference in the plasma selenium concentration between the control and both Adriamycin-treated groups (Figure 5). However, there was a significant difference in the selenium levels when the control

group and selenium-supplemented group (Group II) were compared. A significant difference was also found between the Adriamycin group and the selenium-supplemented group.

Measurement of selenium levels in heart homogenates revealed similar results (Figure 6). The selenium levels from hearts of the animals in the control group were significantly lower, compared with the selenium-supplemented group. Similar results were found between the Adriamycin-treated animals (Group III) and the selenium-supplemented group.

In order to rule out interaction between the antitumor effect of Adriamycin and selenium, we conducted *in vitro* experiments with L_{1210} cells. The cells incubated with Adriamycin and selenium showed the same pattern of inhibition as those incubated with Adriamycin alone, which indicated no alteration of the antiproliferative effect of Adriamycin by selenium (Figure 7). The tumor cell killing by Adriamycin was not altered by the increased tumor cell selenium content as shown in Table 2.

Discussion

The present study established that the rabbit develops cardiomyopathy even after short course of Adriamycin administration. The morphologic changes are identical to those previously reported after chronic administration of the drug in animals and humans.^{3-5,12,16} The reason for choosing short-term Adriamycin administration was to produce early morphologic changes which might be prevented more easily than those observed in chronic administration of the drug.

In the present study we pretreated the animals with selenium, which allowed accumulation of selenium in the heart before Adriamycin reached the myofibers. These experiments differ from those previously reported where Adriamycin and cardioprotectants were administered simultaneously.^{9,12} With this experimental protocol, it appears the Adriamycin does not affect the amount of selenium in the heart tissue (Figure 6). We expected a possible interaction between these two agents. Adriamycin is capable of

forming conjugates with iron and copper.¹⁷ So far, no information is available regarding formation of Adriamycin-selenium conjugates, but such a possibility should be considered. The dose schedule used in the present study differed from that used in previously reported chronic schedules.^{3,4,18} The dose of Adriamycin in this study was slightly higher, but the animals received only four intermittent doses with no deaths observed during the experiment.

The gross examination of the hearts revealed no changes when all experimental groups were compared. The myocardial lesions under light microscopy (data not presented) were identical with those previously described in rabbits.^{4,18}

The major alterations observed in the ultrastructure of the myocardium did not differ substantially from the previously reported findings.^{4,18} The marked loss of myofibrils shown in the hearts of Adriamycin-treated animals (Figure 3) was preserved by selenium supplementation (Figure 4). The sarcoplasmic vacuolation observed in the present study (Figure 3), and considered a prominent sign of Adriamycin-induced

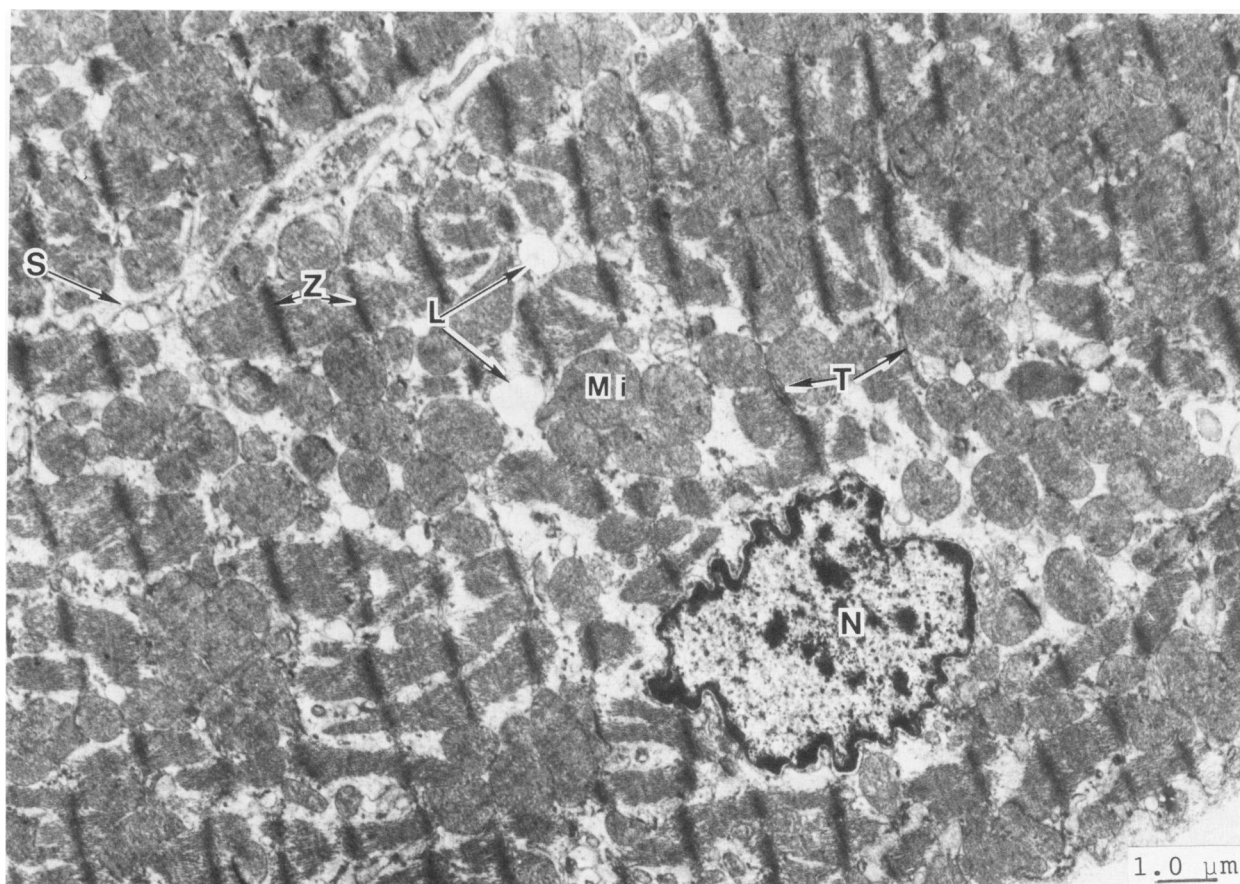


Figure 2—Transmission electron microscopy of myocardium of rabbit after administration of 2 ppm of selenium. (×9000) Myofibrils show good preservation of myofilaments with Z bands at regular intervals. Mitochondria (*Mi*) present in regular rows. *S*, sarcolemma; *T*, tubule; *N*, nucleus; *L*, lipid droplets.

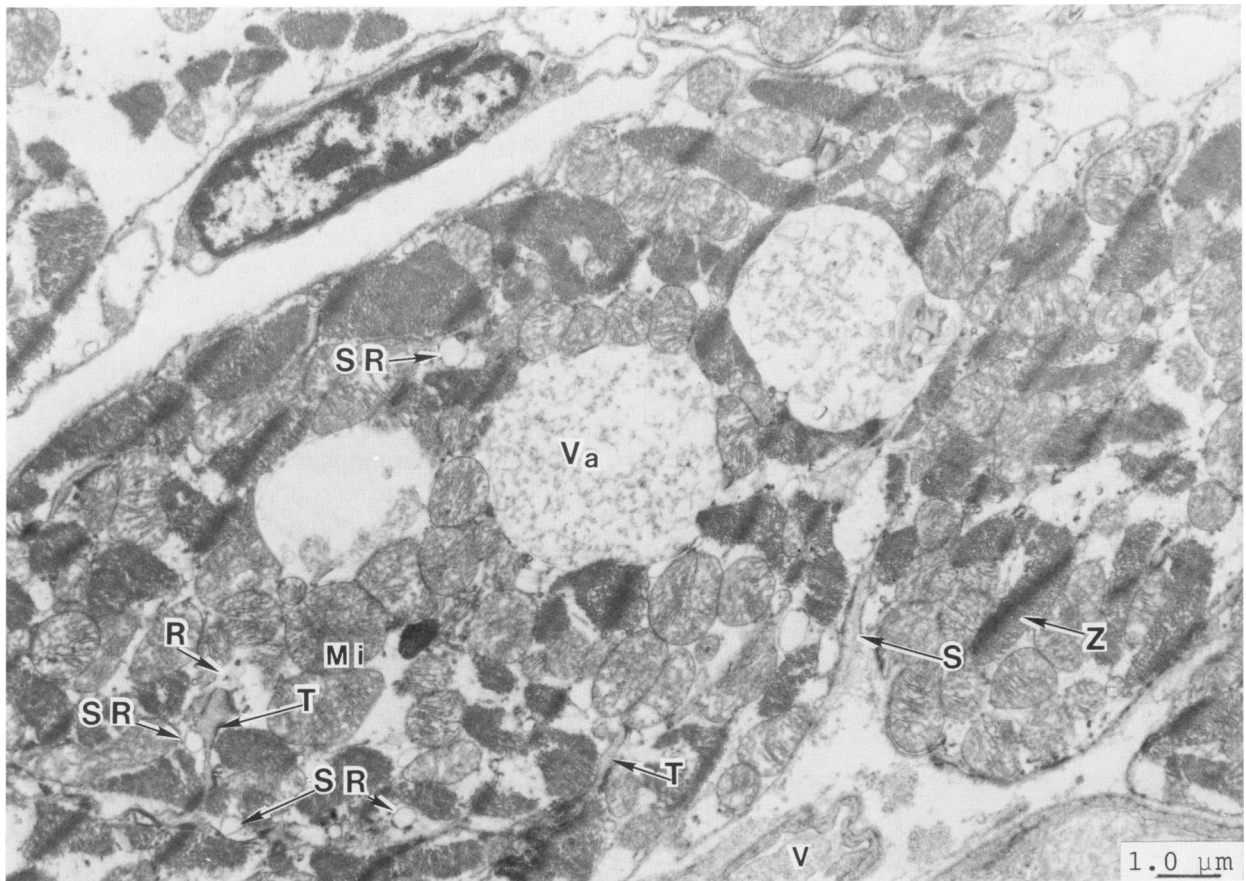


Figure 3—Transmission electron microscopy of myocardium of rabbit after intravenous injections of 3.0 mg Adriamycin/kg of body wt. ($\times 9000$) Marked loss of myofibrils with irregular distribution of Z bands, some of which have few myofilaments attached to them. Dilatation of the sarcoplasmic reticulum (SR) and prominent vacuolation (Va) of myocytes. S, sarcolemma; T, T tubule; V, blood vessel.

myocardiopathy, was not present in the hearts of selenium-treated rabbits (Figure 4). Although the prevention of the heart damage as a result of Adriamycin administration is remarkable, some alterations such as crowding of the mitochondria were still present (Figure 4). Apart from some perinuclear clearing, nuclear alterations were not observed in this study. Such changes have been previously reported in animal and human myocardium after Adriamycin therapy.^{5,19} However, these nuclear changes appear to be related mainly to severe Adriamycin toxicity.⁵ Because the therapeutic schedule used in the present study was short, the morphologic alterations should be considered as early changes. They are similar to those described by Unverferf et al.²⁰

Supplementation with selenium prior to Adriamycin administration prevented occurrence of morphologic changes observed in the animals treated with Adriamycin. Such protection was observed in pilot studies with patients undergoing Adriamycin therapy and pretreated with selenium.²¹ Failure to alter the

incidence or severity of cardiac damage in rabbits and dogs previously reported may be explained by the use of different treatment schedules.^{12,18} Simultaneous administration of Adriamycin and selenium used by these investigators may facilitate drug-drug interaction. This could be at the level of competition for binding sites in the serum and heart or by inactivation of selenium by Adriamycin. In addition, the metabolism of selenium may be different when the micronutrient is utilized by normal myocardium or by myocardium that has been exposed to Adriamycin. Unfortunately, the other studies did not include measurements of selenium levels in the serum or the hearts of the experimental animals, which could provide some explanation of selenium failure. It is of interest that in the experiments of VanVleet et al.,¹⁸ using simultaneous administration of the agents, selenium-treated animals survived longer than the control group, and a moderate decrease in the severity of Adriamycin-induced myocardiopathy was observed.

The mechanism of the protective effect of selenium

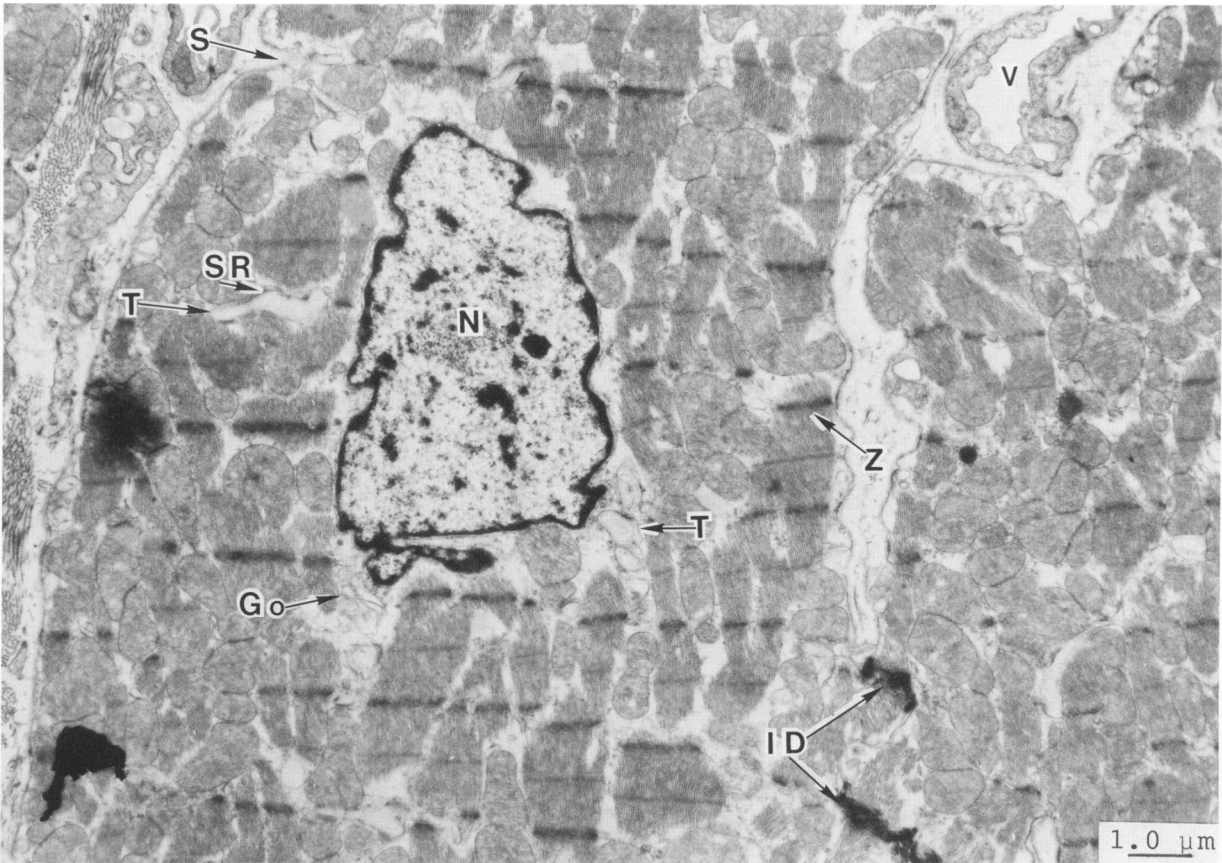


Figure 4 — Transmission electron microscopy of myocardium of rabbit after administration of selenium and Adriamycin. (×9000) Ultrastructural findings are within the limits of normal. S, sarcolemma. Sarcoplasmic reticulum (SR) is seen as a thin channel in close relationship to the T tubule. N, nucleus; Go, Golgi apparatus; ID, intercalated disk; V, blood vessel.

is unclear. Because selenium is considered an active part of glutathione peroxidase, which possesses a high antioxidant activity, it was suggested that the selenium antioxidant effect is indirect through the action of this enzyme.²² In those experiments, the selenium-deficient state was characterized by markedly decreased glutathione peroxidase levels and led to significantly enhanced Adriamycin toxicity at a single intraperitoneal dose of 15 mg/kg. It is of interest to mention that these authors found no decrease in glutathione peroxidase in the liver of the selenium-defi-

cient animals or of the control group. This may indicate some specificity or difference in sensitivity of selenium-dependent glutathione peroxidase in the heart. Revis and Marusic⁷ found that hearts of rabbits treated with Adriamycin contained both reduced selenium concentration and reduced glutathione peroxidase activity. The authors suggested that the decrease in selenium may have been due to an alteration in the selenium flux in the myocardial cell.

In vitro experiments with L₁₂₁₀ cells indicate that selenium did not interfere with the antiproliferative

Table 1 — Quantitative Analysis Performed With Modified Billingham's Grades

Experimental group	Mean score	Ratio no. lesions/ no. blocks	Range
Normal control (I)	0.48	19:40	0-1.0
Selenium alone (II)	0.31	9:30	0-0.5
Adriamycin alone (III)	1.72	34.5:20	1-2.5
Adriamycin + selenium (IV)	0.22	6.5:30	0-1.0

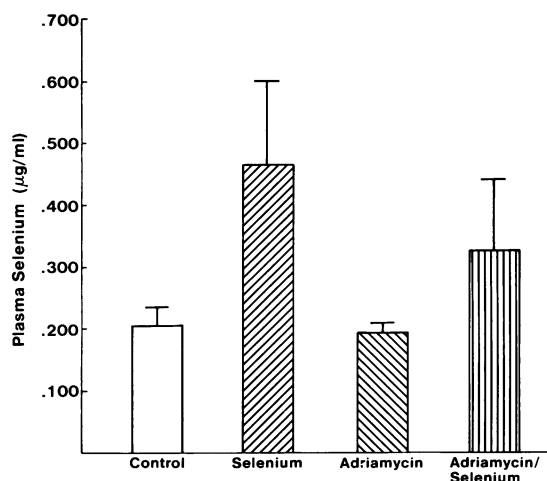


Figure 5—Plasma selenium concentrations of rabbits in all experimental groups.

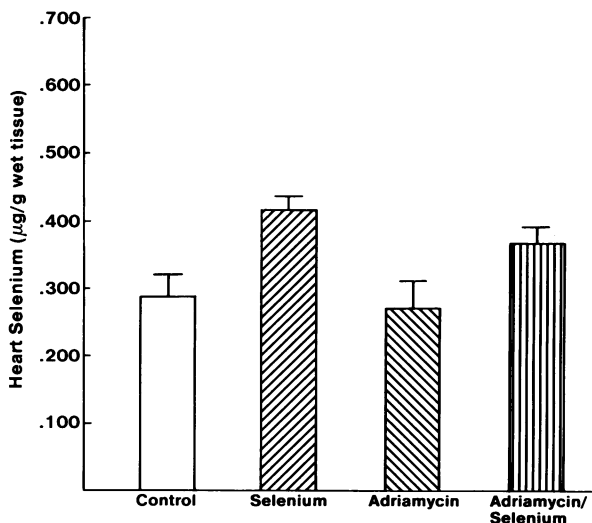


Figure 6—Selenium concentrations in heart tissues of rabbits in all experimental groups.

effect of Adriamycin (Figure 7). Thus, the preventive cardiotoxic effect of selenium would not abrogate the antitumor effect of Adriamycin.

The real mechanism in the selenium–Adriamycin interaction remains unclear regardless of the above mentioned suggestions. The results from the present study indicate that Adriamycin-induced cardiotoxicity could be prevented by selenium if the animals were pretreated with selenium rather than treated simultaneously with both agents. This observation may have some application in preventing this interesting Adriamycin-induced side effect in humans.

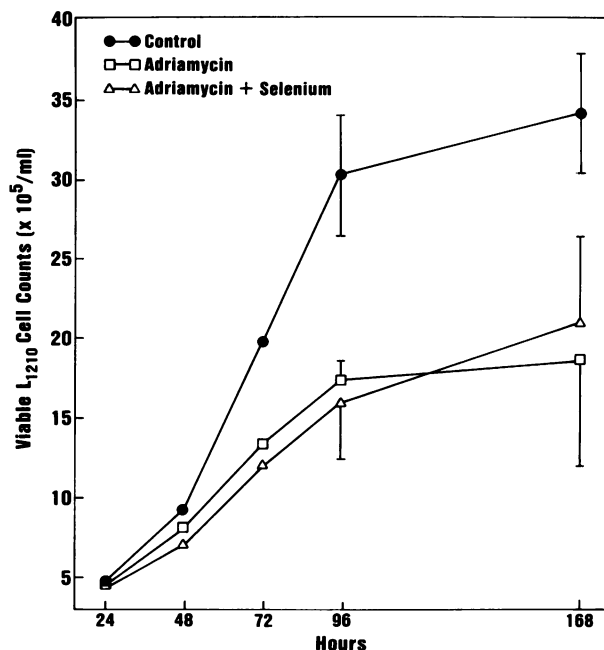


Figure 7—Growth curves of L₁₂₁₀ cells *in vitro* representing control, Adriamycin, and Adriamycin + selenium-treated cultures.

Table 2—Selenium Uptake in L₁₂₁₀ Cells

Sample	Selenium	
	µg/g wet weight	µg/ml
Control L ₁₂₁₀ cells (pellet) (6 × 10 ⁵ /ml)	0.0268	—
Selenium-treated L ₁₂₁₀ cells (pellet) (6 × 10 ⁵ /ml)	0.3736	—
Washed selenium-treated L ₁₂₁₀ cells (pellet) (6 × 10 ⁵ /ml)	0.1205	—
Control medium (supernatant)	—	0.0284
Selenium-treated medium (supernatant)	—	0.2870

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